Available online at www.sciencedirect.com
ScienceDirect
Enzyme and Microbial Technology 41 (2007) 292–295
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Characterization of *Yarrowia lipolytica* and related species for citric acid production from glycerol ☆

William E. Levinson, Cletus P. Kurtzman, Tsung Min Kuo *

Microbial Genomics and Bioprocessing Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, 1815 N. University Street, Peoria, IL 61604, United States

Received 7 October 2006; received in revised form 10 February 2007; accepted 12 February 2007

Abstract

Twenty-seven *Yarrowia lipolytica* strains as well as five strains from three other species of the *Yarrowia* clade (*Aciculoconidium aculeatum*, *Candida hispaniensis* and *Candida bentonensis*) were screened for citric acid production with pure glycerol as the carbon source. The cultures were grown under nitrogen-limited conditions. None of the non-*Yarrowia* strains produced citric acid, although they were able to grow on glycerol. All of the *Y. lipolytica* strains were able to produce citric acid in varying concentrations and, under the screening conditions used, the yields obtained formed a continuum from very low to among the highest reported for this substrate. The highest yielding strain, *Y. lipolytica* NRRL YB-423, produced 21.6 g/L citric acid from 40 g/L glycerol (54% yield). The citric acid to isocitric acid ratio produced by this strain in the initial screen was 11.3, while most of the strains produced ratios of between 2 and 6. Further work on medium optimization with this strain showed that the optimum carbon to nitrogen ratio for the rate of citric acid production was 172 while the best combination of rate and yield was obtained at a C/N ratio of 343. The citric acid to isocitric acid ratios produced reached an optimum at C/N ratios of 343–686.
Published by Elsevier Inc.

Keywords: Citric acid production; Nitrogen limitation; Carbon to nitrogen ratio; *Yarrowia lipolytica*; Glycerol utilization

1. Introduction

Citric acid (CA) is a widely used industrial product. Produced primarily by fermentation, its production volume is second only to industrial ethanol as a fermentation product [1]. While *Aspergillus niger* is the primary organism used for CA production, many yeasts, including *Yarrowia lipolytica*, are known to produce high yields of CA [1]. *Y. lipolytica*, when grown under nutrient-limited conditions, is able to produce CA from a variety of carbon sources, including sugars, alkanes, plant oils, starch hydrolysates, ethanol, and glycerol [1–12].

Conversions of glycerol to value-added products are of increasing interest due to the production of glycerol as a by-product of biodiesel production. While CA production from glycerol by yeasts has been known for many years, it has received little direct study in the peer-reviewed literature until recently [2,3,7]. In the present work, we screened strains of *Y. lipolytica* and related species for the production of CA from glycerol under nitrogen-limited growth. The production of CA by a high-yielding strain with respect to the carbon to nitrogen ratio of the fermentation medium was characterized to show that changes in the carbon to nitrogen ratio affected the ratio of CA to isocitric acid produced and that optimal carbon to nitrogen levels could be defined for the best rate and yield of CA production.

2. Materials and methods

Yeasts screened were from the ARS Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, IL, and were maintained on potato-dextrose-agar slants throughout this study. Citric acid (99+%) was supplied by Fischer Scientific Co. (Pittsburgh, PA). Isocitric acid (ICA) and thiamine hydrochloride were purchased from Sigma–Aldrich (St. Louis, MO). Trypticase-soy broth (TSB) and yeast extract were products of Becton, Dickinson and Co. (Sparks, MD). Pure glycerol (ACS grade) was from Mallinckrodt Baker Inc. (Phillipsburg, NJ) while crude glycerol (∼88%), a by-product of biodiesel production, was supplied courtesy of Biodiesel Systems, LLC (Madison, WI). Other medium components were laboratory grade or better and used without purification.

Initial screening for CA production from glycerol was performed under nitrogen-limited growth conditions and was carried out in a medium with the

☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

* Corresponding author.
E-mail address: kuotm@ncaur.usda.gov (T.M. Kuo).

0141-0229/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.enzmictec.2007.02.005

following composition (g/L in deionized water): glycerol, 40; $(NH_4)_2SO_4$, 0.25; KH_2PO_4, 1.7; Na_2HPO_4, 12; $MgSO_4 \cdot 7H_2O$, 1.25; thiamine hydrochloride, 0.006; yeast extract, 0.25. The C/N (mol/mol) ratio of the medium was 343. Initial pH was 6.0 and sterile bromocresol purple (32 mg/L from a 1000× stock in ethanol) was added post-autoclave as a pH indicator.

Screening was done in single cultures of 25 mL volume in 125-mL Erlenmeyer flasks. The medium was inoculated (1%) from 24-h cultures grown on TSB and growth conditions were 28 °C and 200 rpm. During growth, the pH indicator was used to maintain the pH within a desired range by daily additions of 2 M KOH. Using optical density and pH change as a guide, cultures were incubated for ten days. This appeared to allow most cultures to complete growth. Duplicate samples were taken from each culture at the end of incubation. Cultures were harvested by centrifugation and analysis was performed on the supernatants. The screening experiment resulted in the selection of a high-yielding strain, *Y. lipolytica* NRRL YB-423, for further work. These experiments were performed in duplicate flasks containing 50 mL medium. The screening medium was modified for these experiments as described in Section 3.

Analysis of culture supernatants for CA and glycerol was done by HPLC. CA analysis was performed with an Alltech Prevail Organic Acid column (150 mm × 4.6 mm, 5 μm particle size; Alltech Associates Inc., Deerfield, IL), an isocratic mobile-phase of 0.25% acetic acid and detection at 201 nm with a diode-array detector. Glycerol was analyzed with a Phenomenex Rezex RCU-USP Sugar Alcohol column (250 mm × 4.0 mm; Phenomenex Inc., Torrance, CA), deionized water mobile-phase and Waters 410 Differential Refractometer (Millipore Corp., Billerica, MA). ICA was determined using a commercially available enzyme-based kit (R-Biopharm AG, Darmstadt, Germany). The volumes in the manufacturer's protocol were modified to allow the assay to be performed in microtiter plates.

3. Results and discussion

Twenty-seven *Y. lipolytica* strains, two strains of *A. aculeatum* (NRRL YB-4297 and YB-4298) and three *Candida* strains (*C. hispaniensis* NRRL Y-5579 and NRRL Y-5580 and *C. bentonensis* NRRL YB-2364) were screened for CA production from glycerol (Table 1). The organisms tested were originally derived from a variety of geographic areas and sample sources including industrial and food processing facilities, food products, insects, and environmental and clinical samples. *A. aculeatum* is a member of the *Y. lipolytica* clade [13] and the two *Candida* species have recently been described and placed in this clade as well [14]. Therefore, we included them in our analysis to determine if the ability to produce CA might extend beyond *Y. lipolytica* to other members of the clade. All strains grew on pure glycerol to varying degrees (data not shown). The cultures also required different amounts of base in order to maintain the desired pH range (~pH 4–6) (data not shown).

The *Yarrowia* clade strains of *Aciculoconidium* and *Candida* tested produced no measurable CA under these conditions

Table 1
Strains screened for citric acid production[a]

Species	NRRL accession number	CA (g/L)	ICA (g/L)	CA/ICA ratio	Source
Y. lipolytica	YB-423	21.6	1.9	11.3	Corn wet milling plant
	Y-1094	20.6	4.7	4.4	Unknown
	Y-2180	19.2	6.2	3.1	*Drosophila* sp.
	Y-1095	19.0	3.3	5.8	Unknown
	Y-12975	18.3	11.0	1.7	Clinical
	Y-7677	18.2	7.6	2.4	Unknown
	Y-7208	18.1	3.7	4.9	Refrigerated frankfurter
	Y-1694	18.1	10.5	1.7	Yeast starter culture contaminant
	Y-17536	17.6	6.2	2.8	Vegetable salad
	Y-7817	16.7	5.9	2.8	Canadian bacon
	Y-6795	16.2	3.4	4.7	Soil
	YB-1353	15.8	6.4	2.5	Fly larva
	Y-1093	15.7	6.3	2.5	Unknown
	Y-5386	14.0	3.9	3.6	Petroleum storage tank
	Y-7207	11.4	2.2	5.2	Unknown
	Y-7317	11.3	2.8	4.1	Unknown
	Y-17014	10.4	1.8	5.7	Compost soil
	Y-7484	9.9	1.9	5.2	Clinical, sputum
	Y-11853	9.6	2.2	4.4	Unknown
	Y-6049	9.1	2.9	3.1	Petroleum storage tank
	YB-4681	7.9	1.4	5.8	Soil/feces, rookery
	YB-419	5.5	1.8	3.0	Corn wet milling plant
	YB-523	4.8	2.3	2.1	Corn wet milling plant
	Y-7212	3.6	0.6	6.0	Unknown
	Y-5383	3.3	0.9	3.8	Petroleum storage tank
	Y-7751	3.0	0.6	5.1	Meat
	Y-5476	1.4	0.3	4.7	Unknown
Aciculoconidium aculeatum	YB-4297	0.0	0.0	0.0	*Drosophila pinicola*
	YB-4298	0.0	0.0	0.0	*Drosophila occidentalis*
Candida bentonensis	YB-2364	0.0	0.0	0.0	Apple cider
Candida hispaniensis	Y-5579	0.0	0.4	0.0	Insect larva
	Y-5580	0.0	0.2	0.0	Insect larva

[a] *Y. lipolytica* strains listed in order of high to low CA production. Medium contained 40 g/L glycerol. *Abbreviations*: CA, citric acid; ICA, D-isocitric acid.

(Table 1). The *Y. lipolytica* strains screened produced a continuum of CA concentrations ranging from a high of 21.6 g/L to a low of 1.4 g/L, resulting in a range of yields from 54% to 3.5%. Production levels had a median value of 12.7 and an average of 12.2 g/L CA. The highest yield (54%), produced by *Y. lipolytica* NRRL YB-423, was comparable to that reported for *Y. lipolytica* ACA-DC 50109 (44–56%) utilizing raw glycerol as a substrate [2,3].

A disadvantage of CA production with *Y. lipolytica* is the simultaneous production of the unwanted metabolite ICA, the production of which varies with strain and carbon source [1,5]. Fickers et al. [5] quotes CA/ICA ratios of 9.0 for wild-type *Y. lipolytica* strains grown on glycerol. In our screening experiment, using a medium with a C/N ratio of 343, CA/ICA ratios of between 11.3 and 1.7 were seen, with an average value of 4.0 (Table 1). The majority of the values fell between 2.0 and 6.0. The highest level of CA to ICA achieved in the initial screening experiment was 11.3 and was produced by the highest yielding strain, NRRL YB-423. This was 1.9 times the next highest value of 6.0.

Y. lipolytica NRRL YB-423, which gave the highest yield and best CA/ICA ratio, was selected for further experimentation. Cultures were grown in the base medium with varying amounts of nitrogen and CA production was monitored over time (Fig. 1). All cultures produced CA but those with higher initial nitrogen concentrations utilized this product as incubation progressed, resulting in lower maximum CA production and lower concentrations at harvest (day 11). The highest CA concentration produced (21.8 g/L) was the result of growth at a C/N ratio of 686, giving a 54.5% yield based on the initial 40 g/L glycerol in the medium. The cultures with the same nitrogen concentration as the initial screening medium (3.9 mM; C/N = 343) produced a maximum CA concentration (prior to CA utilization) of 20.8 g/L (52% yield).

The CA production rates achieved during the variable nitrogen experiment are shown in Fig. 2 (versus C/N ratio). The highest rate seen, 134 mg L^{-1} h^{-1}, was in cultures with a C/N ration of 172 (7.8 mM N). Rates were not severely affected by a wide range of C/N ratios, however, there appeared to be a definite optimum in this area. The best combination of rate (116 mg L^{-1} h^{-1}) and yield (52% at day 9) was seen with a C/N ratio of 343 (3.8 mM N), although the maximum production from the 7.8 mM N cultures was probably not captured during the sampling regime for this experiment (Fig. 1). The CA to ICA ratio was also affected by the C/N ratio of the medium with the best CA/ICA ratios, above 9.0, produced at C/N ratios of 343 and 686 (Fig. 3). This data was from day 6 of the experiment, when all cultures were actively producing CA, which may account for the slightly lower CA/ICA ratios seen here versus the initial screening experiment where the data was from the final day of incubation.

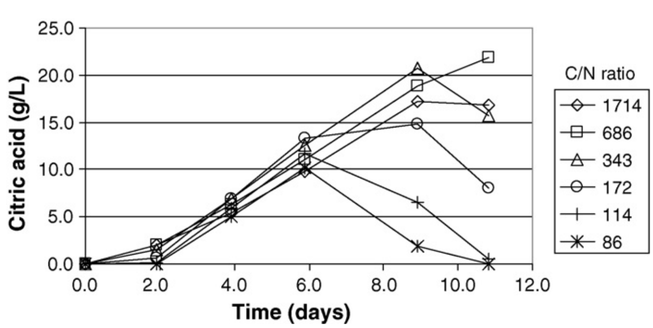

Fig. 1. Citric acid concentration over time in cultures grown with varied initial nitrogen concentrations (C/N ratio). Data are the average of duplicate cultures of *Yarrowia lipolytica* NRRL YB-423.

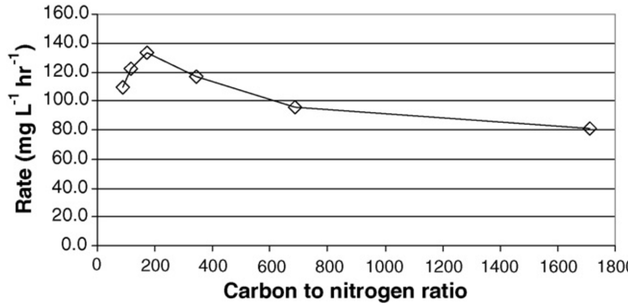

Fig. 2. Citric acid production rate vs. initial nitrogen concentration of the medium (C/N ratio). Rate was measured between day 2 and day 6 of incubation. Data are the average of duplicate cultures of *Y. lipolytica* NRRL YB-423.

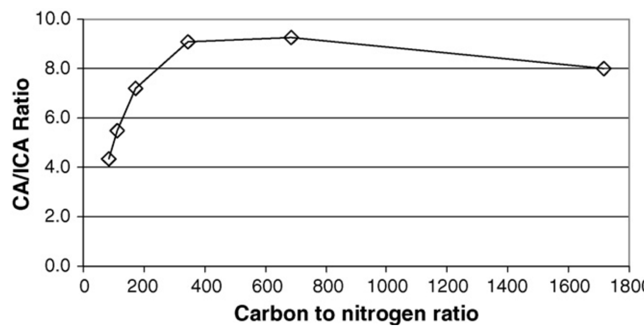

Fig. 3. Citric acid to isocitric acid ratio vs. initial nitrogen concentration of the medium (C/N ratio). Data are from day 6 of incubation. Data are the average of duplicate cultures of *Y. lipolytica* NRRL YB-423.

Citric acid production by *Y. lipoytica* NRRL YB-423 was also tested with crude glycerol that was a biodiesel production by-product (data not shown). The CA yield with this substrate was 55.7% at the time of harvest and the rate of production over a 10-day incubation period was 94 mg L^{-1} h^{-1}, so the yield and production rate with crude glycerol were comparable to that obtained with the pure substrate. Similar CA yields with crude glycerol from a biodiesel process as substrate have been reported for strain *Y. lipolytica* ACA-DC 50109 [2,3].

4. Summary

While CA production by yeasts has been known many years, production using glycerol as a substrate has rarely been the direct focus of published reports. The present work has shown that while CA production from glycerol under nitrogen-limited growth conditions may be a common trait of *Y. lipolytica*, there is significant variability related to this trait and to the associated production of ICA. In addition, the ability to produce CA from glycerol did not extent to the related *Yarrowia*-clade strains tested here.

The highest-yielding strain, *Y. lipolytica* NRRL YB-423, produced CA from glycerol with a yield comparable to that reported in the literature [2,3] while also producing the highest CA/ICA ratio among the strains screened. This work has identified a high-yielding *Y. lipolytica* strain useful for the conversion of glycerol to CA, a value-added product, and has demonstrated the importance of the C/N ratio of the fermentation medium in affecting the CA/ICA ratio of the products produced and in optimizing the rate and yield of CA production from glycerol.

Acknowledgement

The authors wish to thank Eleanor Basehoar-Powers for the verification of the identity of the *Yarrowia lipolytica* strains from sequencing of large subunit ribosomal DNA.

References

[1] Roehr M, Kubicek CP, Kominek J. Citric acid. In: Roehr M, editor. Biotechnology: products of primary metabolism. 2nd ed. VCH Verlagsgesellschaft mbH; 1996. p. 307–45 [Rehm H-J, Reed G, series editor. Biotechnology, vol 6].

[2] Papanikolaou S, Aggelis G. Modelling aspects of the biotechnological valorization of raw glycerol: production of citric acid by *Yarrowia lipolytica* and 1,3-propanediol by *Clostridium butyricum*. J Chem Technol Biot 2003;78:542–7.

[3] Papanikolaou S, Muniglia L, Chevalot I, Aggelis G, Marc I. *Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol. J Appl Microbiol 2002;92:737–44.

[4] Rane KD, Sims KA. Citric acid production by *Yarrowia lipolytica*: effect of nitrogen and biomass concentration on yield and productivity. Biotechnol Lett 1996;18:1139–44.

[5] Fickers P, Benetti PH, Wache Y, Marty A, Mauersberger S, Smit MS, et al. Hydrophobic substrate utilisation by the yeast *Yarrowia lipolytica*, and its potential applications. FEMS Yeast Res 2005;5:527–43.

[6] Finogenova TV, Kamzolova SV, Dedyukhina EG, Shishkanova NV, Il'chenko AP, Morgunov IG, et al. Biosynthesis of citric and isocitric acids from ethanol by mutant *Yarrowia lipolytica* N 1 under continuous cultivation. Appl Microbiol Biotechnol 2002;59:493–500.

[7] Finogenova TV, Morgunov IG, Kamzolova SV, Chernyavskaya OG. Organic acid production by the yeast *Yarrowia lipolytica*: a review of prospects. Appl Biochem Microbiol 2005;V41:418–25.

[8] Crolla A, Kennedy KJ. Optimization of citric acid production from *Candida lipolytica* Y-1095 using *n*-paraffin. J Biotechnol 2001;89:27–40.

[9] Crolla A, Kennedy KJ. Fed-batch production of citric acid by *Candida lipolytica* grown on *n*-paraffins. J Biotechnol 2004;110:73–84.

[10] Venter T, Kock JLF, Botes PJ, Smit MS, Hugo A, Joseph M. Acetate enhances citric acid production by *Yarrowia lipolytica* when grown on sunflower oil. Syst Appl Microbiol 2004;27:135–8.

[11] Aurich A, Forster A, Mauersberger S, Barth G, Stottmeister U. Citric acid production from renewable resources by *Yarrowia lipolytica*. Biotechnol Adv 2003;21:454–5.

[12] Kamzolova SV, Shishkanova NV, Morgunov IG, Finogenova TV. Oxygen requirements for growth and citric acid production of *Yarrowia lipolytica*. FEMS Yeast Res 2003;3:217–22.

[13] Kurtzman CP, Robnett CJ. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Anton Leeuw 1998;73:331–71.

[14] Kurtzman CP. New species and a new combination in the *Hyphopichia* and *Yarrowia* yeast clades. Anton Leeuw 2005;88:121–30.